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c-Myc and Cyclin D3 (*CcnD3*) Genes Are Independent Targets for Glucocorticoid Inhibition of Lymphoid Cell Proliferation[1]

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ABSTRACT

Glucocorticoids inhibit the expression of critical cell cycle-regulatory genes. The G_1 cyclin gene *CcnD3*, which encodes cyclin D3, is inhibited by dexamethasone in P1798 murine T lymphoma cells. Glucocorticoids also inhibit expression of the catalytic partner of cyclin D3, Cdk4. Inhibition of these two genes results in a decrease in the ability to phosphorylate the Rb-1 tumor suppressor gene product. Stable transformation with SV40 T antigen expression vectors prevents glucocorticoid-mediated cell cycle arrest, which is consistent with the conclusion that glucocorticoids inhibit Rb-1 phosphorylation. Overexpression of cyclin D3 suffices to restore Rb-kinase activity in glucocorticoid-treated cells. Nevertheless, overexpression of cyclin D3 does not prevent glucocorticoid inhibition of cell proliferation. Cells transformed with Cdk4 expression vectors, with or without cyclin D3 expression vectors, also undergo G_0 arrest in the presence of dexamethasone. Glucocorticoids inhibit c-*Myc* expression in lymphoid cells, and transient expression of c-Myc protein attenuates the lytic response in glucocorticoid-treated human leukemia cells (R. Thulasi, D. V. Harbour, and E. B. Thompson, J. Biol. Chem., *268:* 18306–16312, 1993). However, P1798 cells stably transfected with c-*Myc* expression vectors are sensitive to glucocorticoid-mediated G_0 arrest. Such transformants withdraw from the cell cycle when treated with dexamethasone. P1798 cells were transformed so as to express both c-Myc protein and cyclin D3 in the presence of glucocorticoids. These Myc/D3 cells continue to proliferate in the presence of dexamethasone, and virtually all of these cells are capable of entering S phase in the presence of the steroid. Rapid apoptotic cell death occurs when wild-type P1798 cells are treated with dexamethasone in serum-free medium. Myc-transformed and cyclin D3-transformed cells also die rapidly when treated with glucocorticoids in the absence of serum. T antigen transformants are resistant to glucocorticoid-mediated apoptosis in serum-free medium. Double transformants that express both cyclin D3 and c-Myc are also resistant to apoptosis in the presence of dexamethasone. We conclude that inhibition of both *CcnD3* and c-*Myc* genes is critical to glucocorticoid-mediated G_0 arrest. Furthermore, those genes that convey resistance to growth arrest also convey resistance to cell death.

INTRODUCTION

Glucocorticoids are important regulators of the development and function of the immune system. These effects are dependent, at least in part, on the ability of glucocorticoids to inhibit proliferation of lymphoid cells. Furthermore, the sensitivity of rapidly proliferating lymphoid cells underlies the widespread use of glucocorticoids as chemotherapeutic agents in the treatment of malignant and benign lymphoproliferative diseases. In spite of the physiological and pharmaceutical importance of glucocorticoids, we remain ignorant of the basic molecular mechanisms that underlie the cellular response to these steroids.

Because the glucocorticoid receptor is a transcription factor, it is reasonable to assume that glucocorticoids regulate the expression of genes that are required for cell proliferation. Glucocorticoids cause G_0 arrest of T lymphoid cells (1, 2), so it is likely that such steroids regulate expression of genes the function of which is essential for progression through G_1. There are several promising candidate genes. Among these, the c-*Myc* proto-oncogene has been particularly interesting. Eastman-Reks and Vedeckis (3) initially observed that triamcinolone acetonide inhibits c-*Myc* expression in murine and human T cell lines. They proposed that this response was central to the antimitogenic effects of glucocorticoids. We and others have pursued this observation.

The abundance of c-Myc mRNA decreases rapidly in glucocorticoid-treated P1798 murine T lymphoma cells (2, 4). Statistically significant inhibition can be observed within 10 min after addition of dexamethasone. P1798 cells subsequently undergo G_0 arrest, which is consistent with the prevailing opinion that expression of c-*Myc* is essential for G_1 progression. Glucocorticoids also inhibit c-*Myc* expression in the human T leukemia cell line CEM-C7 (5). Withdrawal into a G_1 or G_0 state ensues upon glucocorticoid treatment of CEM-C7 cells, and cell cycle arrest is followed by cell death in these human leukemia cells (1). The response in CEM-C7 cells is slower than that observed in P1798 cells, which is consistent with the relative rates at which these two cell lines undergo growth arrest and cell death. Transient expression of c-*Myc* in CEM-C7 cells delays the onset of cell death (6), which is the first direct evidence that c-*Myc* may play a role in glucocorticoid responsiveness of T cells. It has not been determined whether stable transformation with c-*Myc* will make CEM-C7 cells resistant to glucocorticoids.

We have recently begun to study glucocorticoid regulation of the cell division control serine/threonine protein kinases that catalyze the rate-limiting step in progression through G_1. These enzymes are the central focus for growth factor control of cell proliferation (7–10). The G_1 cell division control kinases consist of a regulatory subunit (primarily one of the D cyclins) and a catalytic subunit (a cyclin-dependent kinase, usually Cdk4 or Cdk6). The cyclin D-dependent kinases phosphorylate substrates that, in turn, regulate initiation of S phase. The relevant physiological substrates are not defined, but it is known that cyclin D-dependent kinases phosphorylate the product of the *Rb-1* tumor suppressor gene (11, 12). Phosphorylation of Rb-1 is thought to be essential for progression into S phase (13–15), and there is overwhelming evidence that failure to activate cyclin D/Cdk4 kinase blocks G_1 progression (16, 17). The cyclin D-dependent kinases are activated by every mitogenic hormone tested to date (7, 8). Activity of G_1 cell division control kinases is inhibited by yeast-mating pheromones (18) and by transforming growth factor β (19–21), leading to G_1 arrest of target cells. We have determined that glucocorticoids inhibit the expression of the principal D cyclin in P1798 cells (cyclin D3) as well as Cdk4 (22).

We have studied regulation of cyclin D3 gene (*CcnD3*) expression in some detail[5] under the assumption that inhibition of cyclin D3 must lead to G_1 arrest. The experiments described in this paper were

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[5] Reisman, D. and Thompson, E. A. Glucocorticoid regulation of cyclin D3 (*CcnD3*) gene transcription and mRNA stability in lymphoid cells. Mol Endocrinol., in press.

undertaken to determine whether cyclin D3 is a major target of glucocorticoids and how c-Myc fits into this process. The best characterized substrate of the D cyclin/Cdk kinase complexes is Rb-1, so we initially set out to determine whether glucocorticoid resistance could be obtained if one circumvented tumor suppressor gene regulation of entry into S phase. To this end, we transfected cells with expression vectors for the SV40 large T antigen. T antigen binds to tumor suppressor gene products and obviates the need for their phosphorylation (see Ref. 23 and references therein). Expression of cyclin D3 and/or Cdk4 should be irrelevant in cells that express T antigen. We also transfected cells with vectors that express c-Myc protein and cyclin D3 either alone or in combination. Transformants were analyzed to determine whether any or all of these failed to undergo G_1 arrest in the presence of glucocorticoids.

We also have a long-standing interest in the relationship between glucocorticoid inhibition of cell proliferation and glucocorticoid-induced cell death. The P1798 cell line is particularly advantageous for such studies. P1798 cells undergo reversible G_0 arrest when treated with glucocorticoids in culture in the presence of fetal bovine serum (2). However, P1798 cells undergo a classic apoptotic response when exposed to glucocorticoids in serum-free medium or *in vivo* (24, 25). This provided us with the opportunity to determine whether any of our transformants were resistant to the cytolytic response to glucocorticoids. Our objective is to determine whether the mechanisms of cell death and G_1 arrest diverge in cells that express exogenous cell division control genes.

MATERIALS AND METHODS

Cell Culture. P1798 cells were maintained in RPMI 1640 containing 5% fetal bovine serum and 20 μM 2-mercaptoethanol, as described previously (2). Mid-log phase cultures contained $0.5–1.0 \times 10^6$ cells/ml. As appropriate, cells were exposed to 0.1 μM dexamethasone dissolved in 70% ethanol. Nuclear labeling was achieved by incubating cells in 0.1 μCi/ml [^{3}H]thymidine (approximately 6 Ci/mmol). Labeled cells were washed by centrifugation in PBS and deposited on a microscope slide by cytocentrifugation. The slides were dipped in Kodak NTB2 photographic emulsion and exposed for 3–7 days (26). After development of the emulsion, the cells were stained with Giemsa. To assess nuclear labeling indices, three replicate counts of >50 nuclei were carried out.

As indicated, cells were adapted to growth in serum-free medium composed of RMP1 1640, 25 mM HEPES (pH 7.4), 20 μM 2-mercaptoethanol, 10 mM glucose, 4 mM glutamine, 5 μg/ml transferrin, 5 μg/ml insulin, 5 ng/ml sodium selenite, and 0.5% BSA (24). This adaptation was achieved by transferring cells from medium containing 5% fetal bovine serum progressively into medium containing 2.5, 1.25, 0.6, 0.3, and finally no serum. Cells were subcultured after 3–4 population doublings, and cells in the third to fifth passage after transfer to serum-free medium were used in the experiments described below. Viability was estimated by Trypan blue exclusion (27).

Immunoblotting. Cells were washed in PBS, collected by centrifugation, and lysed for 15 min on ice in lysis buffer, which was composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, with 10 μg/ml each of aprotinin, phenylmethylsulfonyl floride, leupeptin, and pepstatin added just before use. Cell lysates were cleared by centrifugation, and protein concentration was determined. Samples were denatured by boiling for 5 min in 2× Sample buffer containing 0.125 M Tris-HCl (pH 6.8), 1% SDS, 20% glycerol, and 10% 2-mercaptoethanol. Samples (40 μg/lane) were separated by SDS-PAGE on 10–12.5% polyacrylamide gels. Proteins were then transferred to nitrocellulose membrane (Schleicher & Schuell) using a semidry transfer apparatus (Bio-Rad). Membranes were incubated overnight at room temperature in Blotto (50 mM Tris-HCl (pH 7.4)-150 mM NaCl-0.05% Tween 20–5% nonfat dried milk), followed by a 3-h incubation at room temperature in primary antibody diluted in Blotto. Membranes were washed three times in Blotto, followed by a 1-h incubation in goat-antirabbit horseradish peroxidase-conjugated secondary antibody diluted in Blotto. Membranes were washed 3 times in Blotto and developed by enhanced chemiluminescence system (Amersham) and exposed to XAR 5 film (Kodak). The specific protein bands on the autoradiogram were analyzed quantitatively with a Lynx densitometer. The cyclin D3 antibody was a polyclonal rabbit antiserum made against recombinant mouse cyclin D3.[5] The Cdk4 antibody was a gift from Steve Hanks (28). The antibody against SV40 T antigen was purchased from Oncogene Science.

Rb Kinase Assay. Phosphorylation of recombinant GST-Rb was assayed essentially as described in Meyerson and Harlow (29). Approximately 5×10^6 cells were washed in PBS by centrifugation. The cells were lysed by addition of 100 μl of IP buffer (50 mM HEPES (pH 7.5)-150 mM NaCl, 1 mM EDTA-2.5 mM EGTA-10 mM β-glycerophosphate-1 mM sodium fluoride-10% glycerol-0.1 mM sodium orthovanadate-1 mM DTT-10 μg/ml each of aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride), followed by sonication for three 15-s bursts with cooling on ice in between. The sonicated mixtures were clarified by centrifugation at 12,000 × K*g* at 4°C. The protein content of the supernatant fraction was determined, and 500 μg of protein was transferred to a microcentrifuge tube along with 1 μl of polyclonal cyclin D3 antibody. This mixture was incubated for 1–2 h on ice, mixing occasionally. Thereafter, 50 μl of protein A beads were added, and the mixture was incubated for 2–3 h at 4°C with rocking. The beads were sedimented by centrifugation for 30 s at 4°C at 10,000 × *g*. The beads were washed three times with IP buffer and twice with 50 mM HEPES (pH 7.5) containing 1 mM DTT. To the pellet was added 22 μl of kinase buffer containing 1.5 μg recombinant GST-Rb, 10 μCi [γ-^{32}P]ATP (6000Ci/mmol), 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2.5 mM EGTA, 10 mM β-glycerolphosphate, 1 mM sodium fluoride, 1 mM DTT, and 0.1 mM sodium orthovanadate. The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by addition of 25 μl of 2× SDS-PAGE sample buffer (see above), and the samples were processed for electrophoresis on 6% polyacrylamide gels as described above. The gels were dried onto Whatmann 3 MM paper, and autoradiography was carried out.

Transfection. P1798 cells were transfected by electroporation. Approximately 10^7 cells were washed with HBS buffer (containing 20 mM HEPES (pH 7.5), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 5 mM glucose). Cells were suspended in 0.5 ml of HSB containing 10 μg of linearized plasmid DNA. After incubation on ice for 15 min, the cells were electroporated at 1200 V and 25 μF using a BioRad Gene Pulsar. Samples were chilled on ice for 15 min and transferred to flasks containing complete medium. Approximately 90–95% of the cells were killed under these conditions. Two days after electroporation, the cells were exposed to selective agent (either G418 or hygromycin), and the cultures were maintained until exponential growth conditions were reestablished in the presence of the drug. Selection for drug resistance routinely took about 6 weeks. Pooled transfectants were used for all procedures.

Expression vectors were derived from either of two plasmids. The coding sequence of murine c-Myc and murine cyclin D3 were cloned in the "sense" orientation into pMAM/Neo (Clontech), which contains a polylinker site downstream of the mouse mammary tumor virus 3′ long terminal repeat. This vector conveys resistance to G418. The coding sequences of murine c-Myc, murine cyclin D3, and murine Cdk4 were inserted in the sense orientation into the polylinker of pCEP4 (In Vitrogen), which contains the CMV[6] immediate early promoter and the hygromycin-selectable marker. The MMTV/SV40 expression plasmid pD305 was a gift from Jerry Shay (Southwestern Medical Center, Dallas, TX).

RESULTS

Glucocorticoids Inhibit Phosphorylation of Rb-1. Dexamethasone inhibits expression of the genes encoding cyclin D3 and Cdk4, as illustrated in Fig. 1, *A* and *B*, respectively. Cyclin D3 is by far the most abundant G_1 cyclin in P1798 cells.[5] Cyclin D1 protein cannot be detected by immunoblotting, and the corresponding mRNA is undetectable by reverse transcription-PCR. Cyclin D2 protein and mRNA are expressed at levels that are barely above the limits of detection. Inhibition of cyclin D3 expression should therefore be associated with

[6] The abbreviations used are: CMV, cytomegalovirus; MMTV, murine mammary tumor virus.

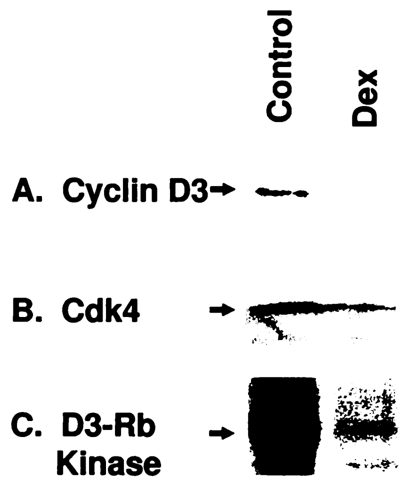

Fig. 1. Glucocorticoids inhibit Rb kinase. Extracts were prepared from mid-log cultures and from cultures that had been treated with dexamethasone for 24 h. Aliquots were denatured, resolved by electrophoresis on polyacrylamide gels, and blotted onto nitrocellulose filters. The filters were probed with antibodies against murine cyclin D3 (*A*) and Cdk4 (*B*), as described in "Materials and Methods." Another aliquot was immunoprecipitated with cyclin D3 antibody. The pellet was incubated with recombinant GST-Rb plus [γ-^{32}P]ATP, as described in "Materials and Methods." The labeled products of this reaction were resolved by electrophoresis and detected by autoradiography (*C*).

a decrease in the ability to phosphorylate the Rb-1 tumor suppressor. A polyclonal antibody against cyclin D3 was used to precipitate cyclin D3-Cdk complexes from mid-log phase and glucocorticoid-treated P1798 cells. These immunoprecipitates were assayed for the ability to phosphorylate recombinant GST-Rb-1 *in vitro*, as shown in Fig. 1*C*. Cyclin D3-Rb kinase activity was reduced in glucocorticoid-treated cells. The extent of inhibition ranged from 75 to 90% in several experiments, although precise quantification of this assay is difficult because of the low signal to noise ratio.

SV40 Large T Antigen Conveys Glucocorticoid Resistance. If glucocorticoid inhibition of P1798 cell proliferation is due to inhibition of Rb-1 phosphorylation, then expression of large T antigen should render P1798 cells resistant to dexamethasone (reviewed in Ref. 23). Cells were transfected with a plasmid vector that contains the coding sequence of the large T antigen of SV40 fused to the MMTV 3′ long terminal repeat. Stable transformants were selected for the ability to proliferate in G418, and G418-resistant populations were tested for glucocorticoid-inducible expression of T antigen. Western blotting data are shown in Fig. 2*A*. Cells that were transformed to G418 resistance by transfection of the vector (pMAM/neo) expressed no detectable T antigen in the presence or absence of glucocorticoids (Fig. 2*A*, *Lanes C* and *D*, respectively). MMTV/T antigen transformants expressed no detectable T antigen in the absence of glucocorticoids (Fig. 2*A*, *Lane C*), but T antigen was induced after addition of dexamethasone (Fig. 2*A*, *Lane D*). Induction of SV40 T antigen in these cells has been shown previously to disrupt Rb-E2F complexes in P1798 cells (30).

In the absence of glucocorticoids, the rate of proliferation of the T antigen transformants was indistinguishable from that of control populations. Wild-type P1798 cells, vector transformants, and MMTV/T antigen transformants all exhibited population-doubling times of 11–14 h in medium containing 5% fetal bovine serum (data not shown). Cells were labeled with [^{3}H]thymidine for 1 h, and nuclear labeling indices were measured to determine the percentage of cells in S phase. All cultures contained 70–80% S-phase cells (data not shown), which is consistent with previous estimates of the duration of S phase in mid-log phase P1798 cultures.

Glucocorticoids cause G_0 arrest of P1798 cells (2). Wild-type

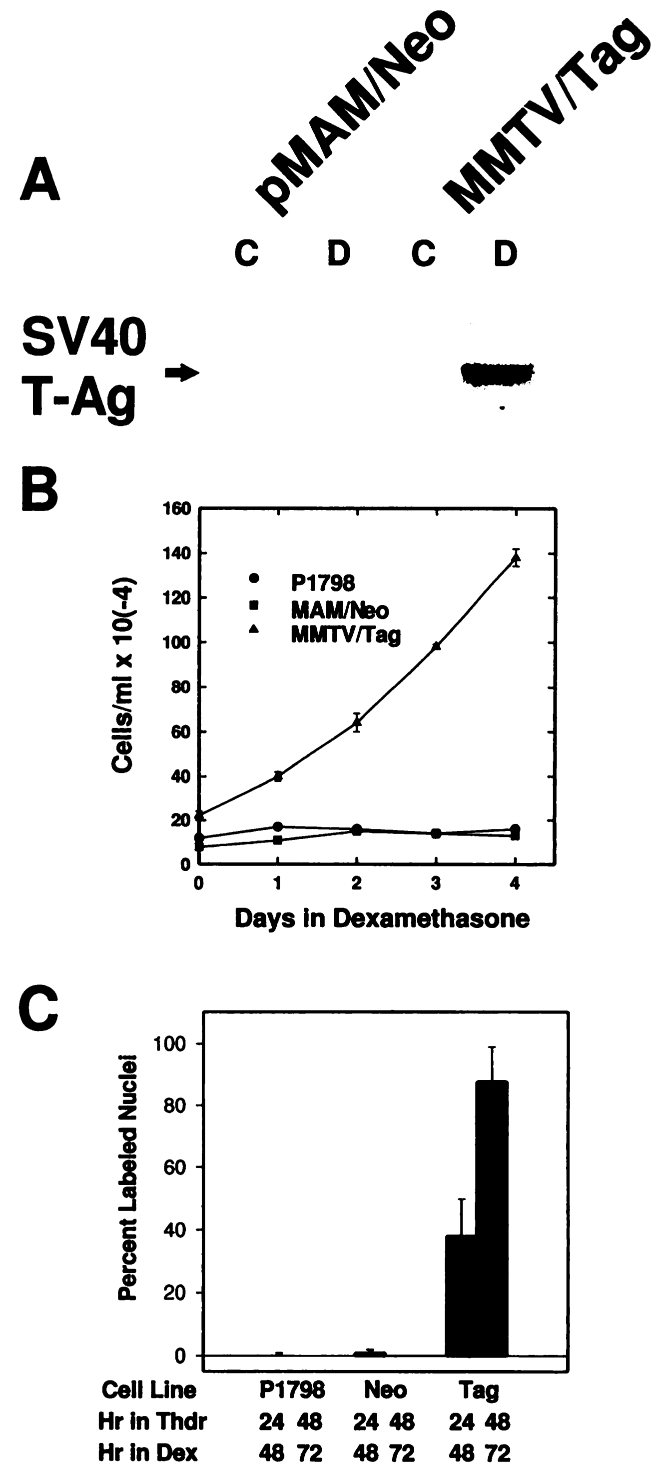

Fig. 2. SV40 T antigen expression renders P1798 cells resistant to glucocorticoids. P1798 cells were transfected with pMAM/neo expression vector or with the MMTV/T antigen expression vector as described in "Materials and Methods." Stable transformants were selected for the ability to grow in medium containing G418. Pooled transformants were treated with dexamethasone for 24 h; proteins were extracted from control (*Lane C*) and glucocorticoid-treated (*Lane D*) cells; and these extracts were assayed for expression of SV40 T antigen (*T-ag*) (*A*), as described in "Materials and Methods" and the legend to Fig. 1. In parallel, pooled transformants were placed in culture in the presence of 0.1 μM dexamethasone (*B*). Cultures were inoculated at initial densities of 15–20 × 10^4 cells/ml, and cells were counted daily thereafter. Viability was assessed by Trypan blue exclusion and was never less than 95% in any of the cultures. *Points*, mean of three parallel, independent cultures; *bars*, SD. In the experiment shown in *C*, wild-type P1798 cells, pMAM/Neo transformants, and SV40 T antigen transformants were treated with dexamethasone for 24 h. Thereafter, [^{3}H]thymidine was added, as described in "Materials and Methods." Cells were harvested 24 and 48 h after addition of labeled thymidine, which corresponds to 48 and 72 h after addition of dexamethasone. Nuclear labeling was measured as described in "Materials and Methods."

populations undergo about one doubling after addition of dexamethasone; the steroid-treated cultures remain stationary thereafter, as shown in Fig. 2*B* (*circles*). P1798 cells do not die when exposed to dexamethasone in the presence of 5% fetal bovine serum (24, 25), so the population density of glucocorticoid-treated wild-type cultures remains constant for several days. P1798 cells that were transformed to G418 resistance by transfection with the pMAM/neo expression vector exhibited properties that were indistinguishable from those of wild-type cells (Fig. 2*B*, *squares*). These cells ceased to proliferate, and culture viability remained high (>95%) for at least 96 h.

Cultures containing MMTV/T antigen transformants did not stop growing when treated with dexamethasone in complete medium (Fig. 2*B*, *triangles*), although the rate of culture growth was significantly lower than that of cultures in mid-log phase growth in the absence of glucocorticoids. Population-doubling times of T antigen transformants in the presence of dexamethasone were 45–50 h, as opposed to 11–12 h for mid-log phase T antigen transformants. Viability of glucocorticoid-treated, T antigen-expressing populations was never less than 95%, as estimated by dye exclusion (data not shown).

Nuclear labeling studies were carried out to assess the heterogeneity of the response of MMTV/T antigen cells to glucocorticoids. Wild-type and T antigen-expressing cells were treated with dexamethasone for 24 h. Thereafter, [^{3}H]thymidine was added to the medium, and nuclear labeling was assayed after 24 and 48 h in the presence of labeled thymidine. The data are shown in Fig. 2*C*. Wild-type cells arrested in G_0 and could not enter S phase, so nuclear labeling of wild-type cells did not occur during the interval from 24 to 48 h after glucocorticoid treatment. However, about one-half of the T antigen-expressing cells initiated S phase during the period from 24 to 48 h after addition of dexamethasone. Almost all of the T antigen-transformed cells were labeled after 48 h in the presence of thymidine (72 h in the presence of dexamethasone), which is consistent with the population-doubling time estimated under these circumstances. The nuclear labeling data indicate that the T antigen-expressing populations are responding in a relatively homogeneous fashion. As far as can be determined from this sort of analysis, almost every G418-resistant cell is also glucocorticoid resistant, insofar as one defines resistance on the basis of G_0 arrest.

Expression of Cyclin D3 and Cdk4 Does Not Convey Glucocorticoid Resistance. If glucocorticoid inhibition of P1798 cell proliferation is exclusively due to inhibition of Rb-1 phosphorylation, then overexpression of cyclin D3 and Cdk4 should render these cell resistant to dexamethasone. P1798 cells were transformed with a plasmid vector that expresses the coding sequence of murine cyclin D3 from the immediate early gene promoter of human CMV. Stable CMV/D3 transformants were selected for resistance to hygromycin. As shown in Fig. 3*A* (CMV/D3, Fig. 3*A*, *Lane C*), maximum expression of cyclin D3 was never more than 2–3 times that observed in untransfected, wild-type populations (P1798; shown in Fig. 3*A*, *Lane C*). Stable CMV/D3 transformants continued to express cyclin D3 in the presence of glucocorticoids (CMV/D3; Fig. 3*A*, *Lane D*). The abundance of cyclin D3 in glucocorticoid-treated populations was similar to that observed in mid-log phase wild-type P1798 cells. The activity of cyclin D3-Rb kinase in glucocorticoid-treated CMV/D3 cells (Fig. 3*B*, *Lane D*) was likewise indistinguishable from that observed in untreated cells (Fig. 3*B*, *Lane C*).

CMV/D3 transformants were assayed for response to glucocorticoids, as shown in Fig. 3*C*. Such cells underwent one net doubling and then arrested without appreciable cell death in the presence of dexamethasone (Fig. 3C, *triangles*). The data indicate that overexpression of cyclin D3 does not suffice for glucocorticoid resistance, even though overexpression of cyclin D3 does appear to restore Rb-1 phosphorylation. Overexpression of Cdk4 either alone or in combination with cyclin D3 does not convey resistance (data not shown).

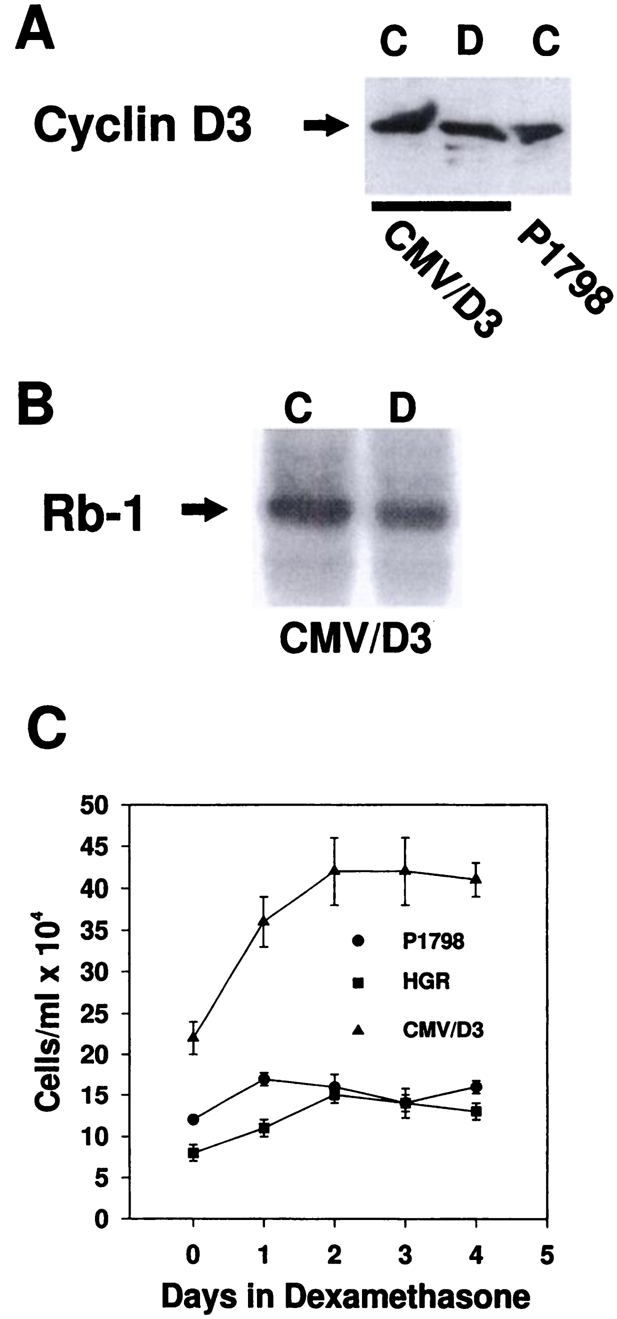

Fig. 3. Overexpression of cyclin D3 restores Rb kinase activity but does not render cells resistant to dexamethasone. P1798 cells were transfected with a cyclin D3 expression vector and stable transformants were selected as described in "Materials and Methods." Extracts were prepared from pooled transformants in mid-log phase growth (*Lane C*) or after 24-h exposure to dexamethasone (*A*, *Lane D*). Extracts were also prepared from control, mid-log phase P1798 cells. Aliquots were analyzed for expression of cyclin D3 by Western blotting as described in "Materials and Methods" and the legend to Fig. 1. In parallel, extracts were assayed for the ability to phosphorylate recombinant GST-Rb (*B*). *Lane C*, immunoprecipitate from control; *Lane D*, from dexamethasone-treated cells. Cell growth of wild-type P1798, pCEP4 vector-transformed, and CMV/D3-transformed cells was measured in the presence of glucocorticoids (*C*). Culture growth and viability were determined as described in "Materials and Methods" and in the legend to Fig. 2.

Overexpression of c-Myc Does Not Convey Resistance to Glucocorticoids. Glucocorticoids inhibit expression of the proto-oncogene c-*Myc* in malignant lymphoid cells (2, 4). In P1798 cells, transcription of c-*Myc* begins to decrease within 5 minutes after addition of dexamethasone (4). Overexpression of c-Myc transiently

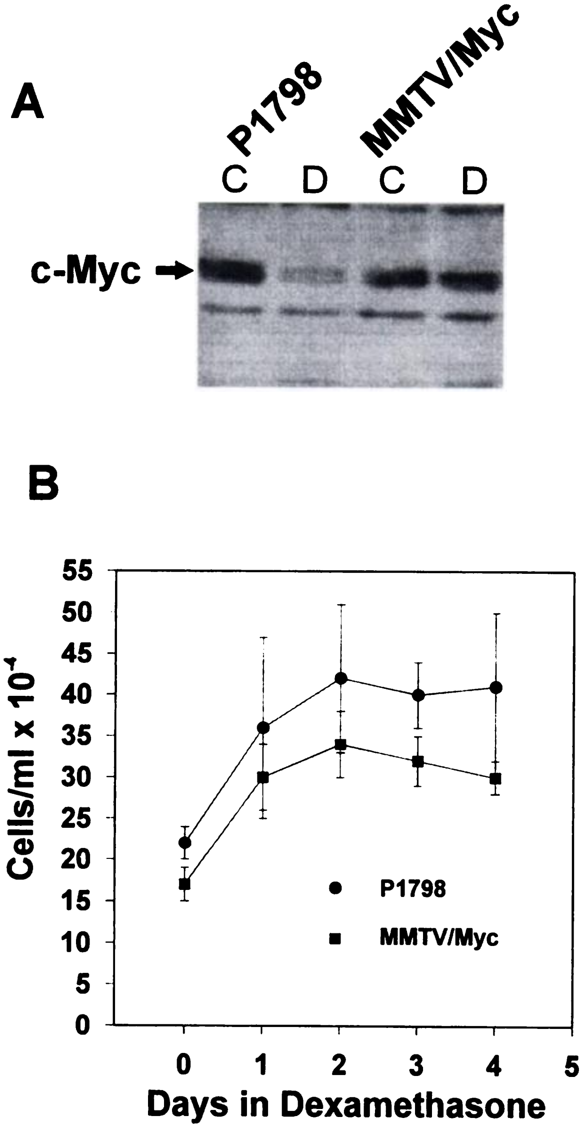

Fig. 4. Overexpression of c-Myc protein does not prevent glucocorticoid-induced G_0 arrest. P1798 cells were transfected with an MMTV/c-Myc expression vector and stable transformants were selected. Pooled transformants were assayed for expression of c-Myc protein (*A*) or for the ability to proliferate in medium containing 0.1 μM dexamethasone (*B*).

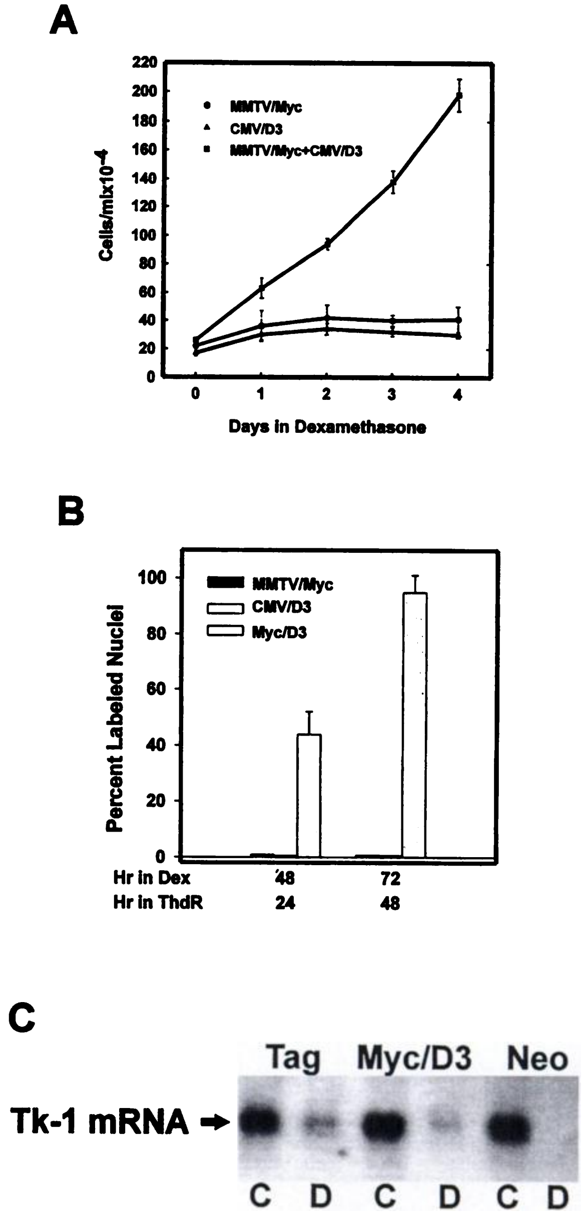

Fig. 5. Overexpression of c-Myc protein and cyclin D3 prevents glucocorticoid-mediated cell cycle arrest. MMTV/Myc transformants, described in the legend to Fig. 6, were transfected with the CMV/D3 expression vector. Stable transformants were selected for the ability to proliferate in G418 plus hygromycin. Pooled transformants were assayed for the ability to proliferate in the presence of 0.1 μM dexamethasone (*A*). In parallel, pooled transformants were treated for 24 h with dexamethasone. [^{3}H]Thymidine was added after 24 h. Cells were removed and assayed for nuclear labeling 24 and 48 h after addition of labeled thymidine (*B*), which corresponds to 48 and 72 h after addition of dexamethasone. In the experiment shown in *C*, MMTV/Tag, Myc/D3, and pMAM/Neo transformants were treated with dexamethasone for 24 h. RNA was extracted from dexamethasone-treated (*Lanes D*) or control, mid-log phase cultures (*Lanes C*). The abundance of Tk-1 mRNA was determined by Northern blot analysis, using 10 μg of total RNA for each lane.

blocks the effects of glucocorticoids in human leukemic CEM-C7 cells (6). Such observations strongly suggest that c-Myc must play an important role in glucocorticoid inhibition of lymphoid cell proliferation.

P1798 cells were transformed with a plasmid in which the coding sequence of murine c-Myc was fused to the MMTV 3′ long terminal repeat. Stable transformants were selected, as described for MMTV/T antigen transformation, and expression of c-Myc protein was assayed in the presence and absence of glucocorticoids. Representative data are shown in Fig. 4*A*. The abundance of c-Myc protein decreased by >75% when wild-type P1798 cells were treated with dexamethasone. The abundance of c-Myc protein in glucocorticoid-treated MMTV/Myc transformants was only slightly less than that observed in untreated cells. Nevertheless, MMTV/Myc transformants were not resistant to glucocorticoids, as shown in Fig. 4*B*.

Overexpression of c-Myc Plus Cyclin D3 Conveys Resistance to Glucocorticoids. A double transformant was made by transfecting the CMV/D3 expression vector into MMTV/Myc cells. These cells were selected for resistance to both G418 and hygromycin. Myc/D3 transformants continued to proliferate in the presence of glucocorticoids, as shown in Fig. 5*A* (*squares*). Population-doubling times of 40–50 h were observed. In the absence of dexamethasone, population-doubling times of about 12 h were observed for Myc/D3 transformants.

Nuclear labeling studies were carried out to determine whether Myc/D3 cells responded to dexamethasone in an homogeneous fashion. Cultures were exposed to dexamethasone for 24 h. Thereafter, [^{3}H]thymidine was added to the dexamethasone-containing medium, and continuous labeling was carried out for an additional 24 or 48 h. The data are shown in Fig. 5*B*. About one-half of the Myc/D3 transformants entered S phase within 24 h after addition of thymidine (48 h after addition of dexamethasone), and all of the cells were labeled within 48 h after addition of the labeled nucleotide. Myc/D3 cells did not enter G_0 in the presence of glucocorticoids. Virtually all of these cells continued to proliferate.

The observation that dexamethasone increases the population-doubling times of Myc/D3-transformed cells is consistent with the conclusion that these cells maintain functional glucocorticoid receptors. Glucocorticoid regulation of the *Tk-1* gene (encoding cytoplasmic thymidine kinase) was measured to confirm receptor function. Dexamethasone reduces Tk-1 mRNA abundance by about 90% in wild-type P1798 cells (31) and in MMTV/Neo transformants (Fig. 5*C*). The abundance of Tk-1 mRNA was reduced 75–85% in T antigen transformants and in Myc/D3 double transformants.

T Antigen, c-Myc, and Cyclin D3 Expression Affects Glucocorticoid-induced Cell Death. Wild-type P1798 cells undergo reversible G_0 arrest when exposed to glucocorticoids in medium containing fetal bovine serum (2); however, wild-type cells die very quickly when exposed to dexamethasone in serum-free medium (24, 25). We undertook a series of experiments to determine whether any of the P1798 transformants described above were resistant to the apoptotic response that is characteristic of P1798 cells treated with dexamethasone in serum-free medium. Cell death under these circumstances is associated with a loss in the ability to exclude vital dyes such as Trypan blue or Cyotdiachrome, and internucleosomal DNA degradation occurs with kinetics that are indistinguishable from those observed for uptake of vital dyes (24, 25). The cells were treated with dexamethasone for 12 or for 24 h, and viability was assessed by dye exclusion. The data are shown in Fig. 6. Control P1798 cells died rapidly. Fifty % cell death obtained within 12 h (Fig. 6*A*). Similar results were obtained with cells that had been transformed to G418 resistance with the pMAM/Neo expression vector. About 90% of these cells were dead within 24 h after addition of dexamethasone (Fig. 6*B*, Neo cells). Expression of Myc protein from MMTV or CMV did not significantly attenuate the lytic response. Forty to 60% death was observed within 12 h (Fig. 6*A*), and 90% death occurred within 24 h after addition of dexamethasone to MMTV/Myc transformants (Fig. 6*B*). Expression of cyclin D3 protein from the CMV promoter did not alter the rate of cell death, and about 50% of these CMV/D3 cells died within 12 h after addition of dexamethasone (Fig. 6*A*).

Cells that express T antigen were resistant to glucocorticoid-induced cell death. No significant decrease in viability was observed within 12 or 24 h after addition of dexamethasone to MMTV/T antigen transformants (Fig. 6, *A* and *B*). The properties of Myc/D3 double transformants were similar to those of T antigen transformants. No significant cell death was observed within 12 (Fig. 6*A*) or 24 (Fig. 6*B*) h after addition of glucocorticoids to Myc/D3 transformants.

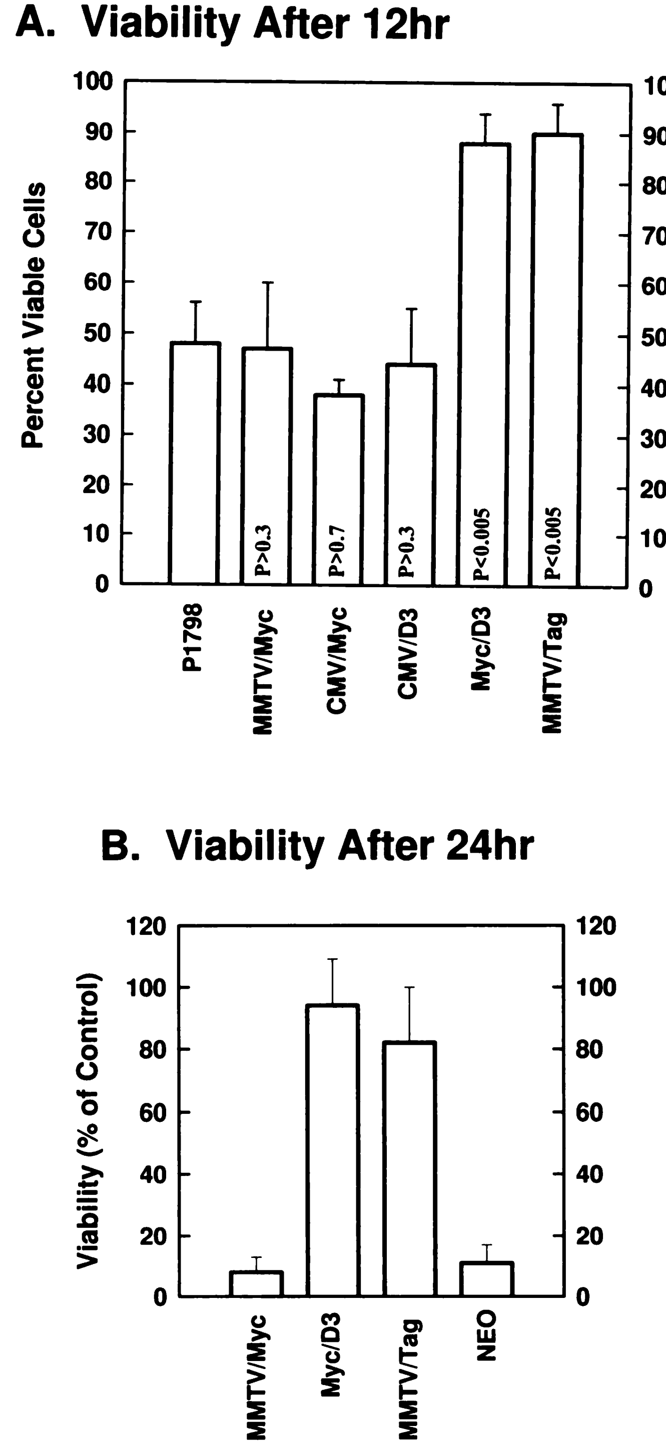

Fig. 6. T antigen (*Tag*) and Myc/D3 transformants are resistant to the lytic effects of dexamethasone in serum-free medium. P1798 cells and transformants were transferred to serum free medium, as described in "Materials and Methods." Dexamethasone was added and viability was assessed by Trypan blue exclusion 12 (*A*) or 24 (*B*) h later. *Points*, mean of three independent, parallel cultures; *bars*, 1 SD. The results are expressed as percent viability. Untreated cultures of all cell lines were 92 ± 4% viable at all time points.

DISCUSSION

Given that glucocorticoids function primarily as regulators of gene expression, and considering the fact that glucocorticoids cause G_1 arrest of lymphoid cells, it is reasonable to propose that glucocorticoids regulate the expression of genes that are required for progression through G_1. The objective is then to identify these putative glucocorticoid-regulated genes. We have attempted to define a set of properties that might reasonably apply to a hypothetical glucocorticoid target gene. We have surveyed the effects of glucocorticoids on a large number of genes that might potentially regulate G_1 progression. We have sought rapid responses that precede withdrawal from the cell cycle and are independent of redistribution within the cell cycle. In this way, we hoped to exclude those genes the expression of which changes as a result of cell cycle arrest. Three potential candidates have been identified: c-*Myc*, *CcnD3*, and *Cdk4*. We have known for some time that the proto-oncogene c-*Myc* is down-regulated rapidly in glucocorticoid-treated cells (2–5). Transient expression of c-*Myc* delays the onset of cell death in glucocorticoid-treated human leukemia cells (6). Furthermore, antisense knockout of c-Myc protein induces cell death in the same cell line. Inhibition of c-*Myc* expression is likely to be important in glucocorticoid regulation of lymphoid cells, and we made and tested several transformants that stably express c-Myc protein. These experiments were done some time ago, and we were puzzled to observe that stable expression of *c-Myc* in P1798 cells had no discernable effect on G_0 arrest or cell death. We concluded that transient and stable expression must be producing different effects, or there was an additional component that was independently regulated by glucocorticoids in P1798 cells.

More recently, we have determined that glucocorticoids rapidly inhibit the expression of two additional G_1 regulatory proteins, cyclin D3[5] and Cdk4 (22). The abundance of both of these proteins changes rapidly in glucocorticoid-treated P1798 cells. Because P1798 cells express no detectable cyclin D1 and very little cyclin D2[5], inhibition of cyclin D3 should deprive the cell of the principal regulatory subunit of the G_1 cell division control kinase complex. Cdk4 is the predominant catalytic component of this kinase complex in fibroblasts and epithelial cells (28). Glucocorticoids therefore inhibit the expression of both the catalytic subunit (Cdk4) and the regulatory subunit (cyclin D3) of a kinase that is known to be essential for G_1 progression. Phosphorylation of tumor suppressor gene products should be blocked, and cyclin kinase inhibitors should be released to inactivate downstream cyclin/Cdk complexes (32, 33).

We attempted to test the hypothesis that down-regulation of cyclin D3 and/or Cdk4 is critical to inhibition of cell proliferation. SV40 T antigen interacts with tumor suppressor gene products so as to obviate the need for their phosphorylation (23). Consequently, expression of T antigen should override inhibition of Rb kinase (*i.e.*, cyclin D3/Cdk4). The observation that T antigen conveys resistance is consistent with the hypothesis that glucocorticoids regulate phosphorylation of tumor suppressor gene products. This conclusion must be advanced with considerable caution, however, because the mechanisms whereby T antigen overrides normal cell cycle regulatory processes are not completely understood.

One must also qualify the conclusion that SV40 T antigen-expressing cells are resistant to glucocorticoids. Wild-type P1798 cells withdraw from the cell cycle in a synchronous fashion upon exposure to dexamethasone. Within 24 h after addition of the steroid, it is virtually impossible to detect cells in S phase. On the other hand, the T antigen transformants do not withdraw from the cell cycle. Within the limits of experimental accuracy, 100% of these cells will continue to divide in the presence of dexamethasone. T antigen completely overrides the G_0 blockade. However, T antigen does not completely override other effects of glucocorticoids. The T antigen transformants proliferate much more slowly in the presence of dexamethasone, suggesting that other factors may also contribute to the glucocorticoid sensitive phenotype.

Although SV40 T antigen is sufficient to block G_0 arrest, this effect is not solely due to overriding the inhibition of cyclin D3/Cdk4 kinase. Ectopic expression of cyclin D3 can restore Rb kinase activity, at least *in vitro*, but this does not suffice to make the cells resistant to glucocorticoids. Our initial assumption was that this might be due to simultaneous inhibition of Cdk4 and cyclin D3. However, double transformants that express both cyclin D3 and Cdk4 were as sensitive to glucocorticoids as are wild-type cells or cells transformed with cyclin D3 alone. In retrospect, the lack of effect of Cdk4 might have been anticipated. It has recently been suggested that Cdk6 may be the principal catalytic subunit of cyclin D kinases in lymphocytes (28). Cdk6 is much more abundant than Cdk4 in P1798 cells, and Cdk6 is not inhibited by glucocorticoids (22). Our data are consistent with the conclusion that cyclin D3/Cdk6 is the principal Rb kinase in P1798 cells. Cdk4 may have other functions that are not essential for G_1 progression.

Double transformants that express both cyclin D3 and Myc proteins are as resistant as SV40 T antigen transformants. G_0 arrest does not occur in these cells. The data suggest that cyclin D3 and c-Myc proteins have parallel, independent functions in cell cycle progression. Both genes are inhibited by glucocorticoids, and both responses appear to contribute to G_0 arrest. The population-doubling times of D3/Myc transformants are significantly increased in the presence of dexamethasone. This observation suggests that different factors may govern the commitment to divide and the rate at which division can occur. It is known that glucocorticoids inhibit glucose uptake and translational efficiency in lymphoid cells (34, 35), and such metabolic effects may limit the rate at which glucocorticoid-treated P1798 cells can divide. Alternatively, inhibition of Cdk4 expression may prolong G_1 without causing G_1 arrest.

There is a long-standing interest in the relationship (if any) between inhibition of cell proliferation and cell death among lymphoid cells. Harmon *et al.* (1) first observed that G_1 arrest precedes cell death in human leukemia cells. P1798 cells undergo reversible G_0 arrest when treated with dexamethasone in medium containing fetal bovine serum (2, 24), indicating that growth arrest does not irreversibly entrain apoptosis. It has also been shown that overexpression of *Bcl2* prevents cell death but does not block inhibition of lymphoma cell proliferation (36). Although firm data have not been forthcoming, we have long proposed that G_0 arrest and cell death were likely to reflect independent pathways (24). The data presented above are not consistent with this notion. Transformed cells that do not undergo G_0 arrest are also resistant to apoptosis in serum-free medium. The significance of this is unclear at this time, and we are hesitant to conclude that genes such as *c-Myc* and *CcnD3* are directly involved in cell death. Nevertheless, the data strongly hint at a direct relationship between cell proliferation and cell death in glucocorticoid-treated lymphoma cells, much as originally proposed by Harmon *et al.* (1). The nature of this relationship requires additional analysis.

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